Regulatory Role of Retinoic Acid on Cultured Mouse Keratinocyte Inositol Phospholipid Metabolism: Dose-dependent Release of Inositol Triphosphate

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The incorporation of precursor ^{14}C-myoinositol into the three cellular inositol phospholipids (PtdIns, PtdInsP, and PtdInsP$_2$) of cultured, rapidly proliferating keratinocytes is significantly enhanced by the exogenous addition of a high concentration (1×10^{-7} M) of all-trans retinoic acid or its analog 13-Cis analog, whereas a similar incubation with a low concentration (1×10^{-10} M) of the same retinoid resulted in an insignificant incorporation of the radio-precursor into the three inositol phospholipids. Incorporation was most marked into the more phosphorylated PtdIns4P and PtdIns4,5P$_2$. These results indicate that retinoic acid affects the biosynthesis of the inositol phospholipids at high concentrations. In contrast, the hydrolysis of ^{14}C-PtdIns4,5P$_2$ and release of ^{14}C-InsP$_3$ from the prelabeled keratinocytes is markedly enhanced by a low physiologic concentration (1×10^{-10} M) of retinoic acid or its 13-Cis analog. The hydrolysis is rapid, with an accompanying elevated transient release of ^{14}C-InsP$_3$. High concentration (1×10^{-5} M), on the other hand, supresses ^{14}C-InsP$_3$ release. These results taken together underscore a bifunctional, dose-dependent effect of both the all-trans-RA and its 13-Cis analog on the synthesis and hydrolysis of keratinocyte PtdIns4,5P$_2$. Furthermore, the results suggest that at low physiologic concentrations, these retinoids may function as agonists to perturb the membrane resulting in induced rapid hydrolysis of cellular PtdIns4,5P$_2$, which is coupled to a "transient" generation of InsP$_3$ (an intracellular second messenger). The rapid formation of this putative "second messenger" may in turn play a role in the cellular proliferative or differentiating biochemical events in the murine keratinocytes. *J Invest Dermatol 92;72–77, 1989*

The early recognition that retinoids (natural and synthetic analogs of retinoic acid) may be involved in the regulation of epithelial differentiation evolved from the observations of Wolbach and Howe [1], who reported that vitamin A deficiency results in squamous metaplasia and keratinization of epithelial tissue. This observation was followed by another equally significant observation that excess vitamin A inhibited keratinization of chick embryo skin, transforming it into a mucus-secreting epithelium [2]. Many investigations since that time have attempted to elucidate the role of the retinoids in epithelial differentiation in tissue models, in organ culture, and in isolated epidermal cells (keratinocytes) [3–12]. These investigations have generated conflicting results, presumably because of the vast difference in concentrations of retinoids employed in these studies.

The first attempt to determine the molecular mechanism of supressed keratinization by retinoids using tissue culture techniques was described by Yuspa and Harris [3]. These authors reported that the addition of 12.5 μg/ml of retinyl acetate to cultured cells altered differentiation by inhibiting desmosome and tonofilament formation. These changes were accompanied by increases in glycoprotein synthesis and inhibition of DNA synthesis. In contrast to the above reported growth inhibition, Christophers [4] reported the stimulation of cultured guinea pig epidermal cells after the addition of 10 μg/ml of retinoic acid to culture medium. The discrepancy in results from these studies and others clearly underscore the significance of varying concentrations of retinoic acid [RA] when evaluating retinoic acid's modulatory effects on epidermal cell differentiation and proliferation. It also emphasizes the need to delineate the effects of these retinoids at low physiologic concentrations and at high pharmacologic concentrations. Although the modulatory effects of retinoic acid and its synthetic analogs (retinoids) have been evaluated in vitro on epithelial differentiation [5–10] and on proliferation [11,12] in various systems, the regulatory role of this vitamin acid at physiologic concentrations on membrane biochemical events, which is quite distinct from its pharmacological

Manuscript received September 30, 1987; accepted for publication June 13, 1988.

This work is supported in part by Research Grant AM-30679 and AM26009 of the U.S. Public Health Service. Dr. Isseroff is the recipient of Clinical Investigatorship Award AM01195 of the National Institutes of Health.

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Abbreviations:
$CHCl_3/CH_3OH$: Chloroform/methanol
DAG: Diacylglyceride
DMEM: Dulbecco modified Eagles's medium
FCS: fetal calf serum
LiCl: lithium chloride
InsP: inositol phosphate
InsP$_2$: inositol bisphosphate
InsP$_3$: inositol triphosphate
PtdIns: phosphatidylinositol
PtdIns4P(PtdInsP): phosphatidylinositol 4-phosphate
PtdIns4,5P$_2$(PtdInsP$_2$): phosphatidylinositol 4,5-bisphosphate
PA: phosphatidic acid
RA: retinoic acid
TLC: thin-layer chromatography
UV: ultraviolet

0022-202X/89/$03.50

role and may influence the maturation of the epidermal cells, is still unclear.

To explore a possible retinoic acid-induced membrane regulatory role, we investigated a rapidly recognizable phenomenon whereby the early responses of cells to an external stimulus (agonist) has been reported to involve the rapid hydrolysis of the inositol phospholipids, particularly the phosphatidylinositol 4,5-bisphosphate (PtdIns4,5P_2). This hydrolysis is now recognized to result in the generation of inositol 1,4,5-triphosphate (InsP_3) and 1,2-diacylglycerol (DAG), both described as "second messengers." These putative messengers in turn are reported to mediate either cell proliferation or differentiation through signal transduction [13,14]. The role of retinoic acid at physiologic levels on the early biochemical events in keratinocyte membranes is unclear, and we found that a close relationship exists between the hydrolysis of PtdIns4,5P_2, the generation of InsP_3, and the subsequent onset of differentiation of normal primary culture of murine keratinocytes [22]. Because of these findings we tested whether retinoic acid and its 13-Cis analog at low physiologic concentrations exert any preferential biologic actions on the membrane phospholipids of these cells via the rapid hydrolysis of the cellular membrane PtdIns4,5P_2 coupled with the intracellular accumulation of InsP_3.

MATERIALS AND METHODS

Reagents and animals Neonatal Balb/C mice were purchased from Simonsen Laboratory (Gilroy, CA). Dulbecco's Modified Eagles' Medium was obtained from Flow Laboratories, VA. Fetal calf serum was from Irvine Scientific (Irvine, CA). Myo-(U-^{14}C)-inositol (333mCi/mmol) was purchased from Amersham (Arlington Heights, IL). Retinal (vitamin A aldehyde) and lithium chloride were purchased from Sigma (St. Louis, MO.). Anion exchange gel AG1-X4 was purchased from Bio-Rad Inc. (Richmond, CA). All-trans and 13-cis-retinoic acids were generous gifts from Hoffman LaRoche (Nutley, New Jersey). All other chemicals were reagent grade (Fisher Scientific, Santa Clara, CA).

Epidermal Keratinocyte Cultures Mouse epidermal cells were isolated from neonatal Balb/C mice and used to establish a primary culture of keratinocytes according to the procedure of Yuspa and Harris [3]. Briefly, mice were sterilized in ethanol and subsequently anesthetized on ice for 30 min. Epidermal sheets were separated from whole skin by floatation in 0.25% trypsin in Hank's balance salt solution overnight at 4°C. The detached epidermal pieces were minced and stirred to obtain single cell suspension. After filtration, viability of the cells was ascertained by the trypan blue dye exclusion technique before plating the cells onto 35-mm petri dishes at an initial cell density of 2.2×10^6/35 mm plate. The cells were cultivated in Dulbecco's Modified Eagle's Medium (DMEM) (Flow Laboratories, VA) supplemented with 10% fetal calf serum (Irvine Scientific, CA) for 37°C with 5% CO_2. These cells grow initially 0–36 h after plating as a monolayer characterized by a population of primarily proliferating cells similar to those of the basal layer of the epidermis. After approximately 36 h, characteristics of differentiation appear as assessed by two criteria: i) morphologically by the presence of cornified envelopes (squames), which were assayed according to the procedure described by Sun and Green; and ii) biochemically by the increased activity of the serine protease plasminogen activator in the cell lysates, which were assayed according to the procedure described by Isseroff et al [23]. This primary culture of murine keratinocytes, therefore, provides a continuous culture system for investigating the phenomena of both proliferation and differentiation.

Long-Term Effect of All-trans RA on the Incorporation of [^{14}C] Inositol into Inositol Phospholipids of Keratinocytes Cultured in Fresh Fetal Calf (Retinol-Containing) Serum In initial experiments we tested the effects of all-trans-RA on the incorporation (biosynthesis) of free-radioinositol into the inositol phospholipids. Briefly, at the initial plating of the cells, high (1×10^{-7} M) or a low (1×10^{-10} M) concentration of this retinoid was added to two separate plates of keratinocytes being cultured in a medium containing fresh fetal (retinol-containing) serum and myo-[U-^{14}C]inositol (4.4×10^6 cpm). The fresh fetal calf (retinol-containing) serum is used in standard primary cultures. Control cells, though similarly cultured, contained no exogenously added retinoic acid. In some experiments, to substantiate the specificity of the all-trans-RA effect another parallel control incubation was carried out which contained retinol (a vitamin A aldehyde) with no defined biologic effect on these cells. Cells were incubated for long hours (12, 24, and 36 h, respectively). The time 0–36 h after plating was taken as the "proliferative mode" of the keratinocyte growth based on a previous determination from this laboratory [22]. The establishment of this time period was based on morphologic evaluation of the keratinocytes after assaying for squame cell formation at various time periods and secondly, after biochemical estimation of the activity of the serine protease plasminogen activator in the cell lysates [23]. At the end of these designated time points, the excess free [^{14}C]inositol contained in the medium of each plate was aspirated off and the [^{14}C]labeled cells washed three times with phosphate buffered saline. The washed cells were scraped from the petri dish in methanol and homogenized in acidified $CHCl_3/CH_3OH$ (2:1, v/v). Separation of the extracted [^{14}C]labeled inositol phospholipids was carried out as described under the section entitled *Extraction and Analysis of the [^{14}C]labeled Inositol Phospholipids.*

Long-term Effect of All-trans-RA on the Incorporation of [^{14}C] Inositol into Inositol Phospholipids of Keratinocytes Cultured in UV-Irradiated (Retinol-Free) Serum In separate experiments, similar concentrations of all-trans-RA as indicated above were incubated with cells cultured in a medium supplemented with UV-irradiated (retinol-free) serum. Rationale for incubation in a retinol-free medium is based on the reported presence of retinol (precursor of retinoic acid) in fresh serum which could dilute the exogenously added retinoic acid. To obtain the retinol-free serum, fresh fetal calf retinol-containing serum was irradiated at 254 nm for 30 min prior to being used for incubations. This UV-irradiated treatment has previously been reported to inactivate serum retinol derivatives [15,16], thereby removing from the incubation mixture endogenous retinol as substrate that could undergo transformation into retinoic acid. To ascertain whether or not the serum retinol was inactivated by the UV-irradiation we determined and confirmed the disappearance of characteristic retinol UV-absorption spectrum at 325 nm from the irradiated serum (DeLuca, personal communication). The irradiated fetal calf serum is designated as "retinol-free" serum and was used in incubations as indicated. Extractions after incubations and identification of products are as described in the text.

Effect of All-trans-RA on the Hydrolysis of Keratinocyte [^{14}C]PtdIns4,5P_2 in Medium Supplemented with Either Retinol-Containing Serum or UV-Irradiated (Retinol-Free) Serum To determine the effects of varying concentrations of all-trans RA on the hydrolysis of keratinocyte PtdIns4,5P_2 (an important hydrolytic step which results in the generation of "second messengers"), the cells were initially prelabeled to equilibrium with 4.4×10^6 cpm [^{14}C]inositol for 36 h in DMEM that was supplemented with fresh (retinol-containing) serum as described in the preceding paragraph. The medium was siphoned off and replaced with either fresh retinol-containing calf serum or with UV-irradiated (retinol-free) serum. The effects of both retinoids were tested, respectively, in an incubation medium that contained either a low concentration (1×10^{-10} M) of all-trans-RA or its 13-Cis-analog as indicated. LiCl (10 mM) was added to each incubation to inhibit the activity of the myo-inositol-1-Phosphomonesterase. Two control incubations were similarly treated and contained either no exogenous retinoic acid or retinal. Hydrolysis of the [^{14}C]inositol phospholipids in the prelabeled cells was evaluated at short time periods (0, 30, 60 and 120 sec). A schematic representation of the experimental protocol used in these studies is shown in Fig 1. At the end of each incubation, the [^{14}C]-labeled cells were scraped off the dishes and the cells homogenized in organic solvent as described above. Similar experimental protocols were followed in

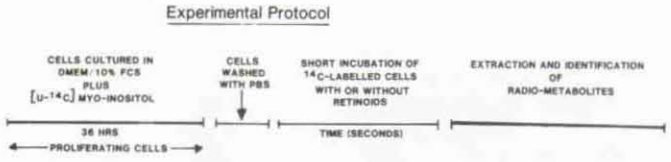

Figure 1. A Schematic Representation of the Experimental Protocol.

subsequent studies to test the effects at higher concentrations (10^{-7} M or 10^{-5} M) of both all-trans-RA and 13-Cis-RA on the cells.

Extraction and Identification of the [^{14}C]-labeled Inositol Phospholipids and the Water Soluble ^{14}C-Inositol Phosphates Total [^{14}C]labeled polyPtdIns was extracted with acidified $CHCl_3$: CH_3OH (2 : 1, v/v). The mixture of [^{14}C]-PtdIns extracted into the organic solvent was spotted on precoated silica gel G plates and separated in the solvent system of chloroform : methanol : 20% methylamine (60 : 36 : 10, v/v). Radioactivity on each plate was scanned and analyzed by the Berthold Automatic TLC-linear Analyzer (Model LB 2832), equipped with an Apple IIe computer.

The [^{14}C]inositol phosphates contained in the aqueous supernatant extracts were percolated onto an anion exchange chromatographic column (AG1-X4, Bio-Rad) as described by Berridge et al [21]. Briefly, the aqueous phase, which contained [^{14}C]radioactive hydrolytic products, was neutralized with 0.1N NaOH to pH 7-8, and was then applied to a column (0.9 cm i.d.) containing 1.5 ml slurry of anion exchange gel, AG1-X4 formate (200–400 mesh), which was obtained from Bio-Rad Laboratories (Richmond,CA). The column was first eluted with 20 ml of H_2O to remove free [^{14}C]inositol. Subsequent fractions were eluted and collected with i) 20 ml of buffer containing 5 mM sodium tetraborate and 60 mM sodium formate (pH 9.0), which elutes the [^{14}C]glycero-phosphoinositol; ii) 16 ml of buffer containing 0.2 M sodium formate in 0.1 M formic acid, which elutes [^{14}C]inositol phosphate (InsP); iii) 16 ml of 0.4 M sodium formate in 0.1 M formic acid, which elutes the [^{14}C]inositol bisphosphate ($InsP_2$); and iv) 10 ml of 1.0 M sodium formate in 0.1 M formic acid, which elutes [^{14}C]inositol triphosphate ($InsP_3$). Aliquots were taken from each fraction and radioactivity was determined by counting in a liquid scintillation counter using Redisolv (Beckman) as the scintillation cocktail.

Phosphorous Assay Phosphorus assay of the total cellular extract was done according to the method of Rouser [24]. Sodium phosphate was used as the standard.

Statistical Analysis Standard statistical methods were used to determine the mean values and SEM. Student's T-test was used for comparison between the mean of the observations. The probability (p) that statistical significance was reached was determined at levels of 0.05 and below.

RESULTS

Dose-dependent Effect of All-trans-RA on the Incorporation of [^{14}C]Inositol into Keratinocyte Inositol Phospholipids Cultured in Medium Supplemented with Fresh (Retinol-Containing) Serum Data in Fig 2 revealed that Myo-[U-^{14}C] inositol was linearly incorporated into the three inositol phospholipids with or without retinoic acid during the first 36 h after plating the keratinocytes in a culture medium supplemented with fresh (retinol-containing) serum. Control cultures contained no exogenously added retinoid. Morphologic evaluations of the keratinocytes previously determined during this 36-h period revealed that these cells were viable, mostly basaloid, and in the proliferative mode (data not shown).

In the presence of added exogenous all-trans-RA, incorporation of [^{14}C]inositol into the keratinocyte was altered. For instance, when incubated with a high concentration of all-trans-RA (1×10^{-7} M), the incorporation of [^{14}C]inositol into all three inositol phospholipids was significantly ($p < 0.004$) enhanced when compared to non-retinoid containing incubations. This increased incorporation of precursor ^{14}C-inositol was particularly marked in the most highly phosphorylated [^{14}C]PtdIns4,5P_2 (a substrate for the generation of the putative "second messengers"). In contrast, when the cells were incubated with a low concentration of all-trans-RA (1×10^{-10} M) the incorporation of [^{14}C]-inositol into [^{14}C]PtdIns4,5P_2 over 36 h, although evident, was overall less marked when compared to the higher concentration of 1×10^{-7} M. The effects of all-trans-RA on the incorporation of precursor ^{14}C-inositol into the less phosphorylated ^{14}C-PtdIns4P and ^{14}C-PtdIns were less consistent and clear. This is probably due to the unknown effects of the retinoids on the two kinases that catalyze the phosphorylation of PtdIns into PtdIns4P and PtdIns4P into PtdIns4,5P_2.

Comparison of the Effects of All-trans RA on [^{14}C] Inositol Incorporation into Keratinocyte ^{14}C-PtdIns4,5P_2 Cultured in either Retinol Containing or Retinol-free Medium The capacity of all-trans-RA to enhance the incorporation of free inositol into PtdIns4,5P_2, prompted us to focus on the biosynthesis of only this highly phosphorylated PtdIns4,5P_2. Thus, a comparison was made on the effects of all-trans retinoic acid in a fresh serum retinol-containing medium (A) and a medium supplemented with UV-irradiated (retinol-free) serum (B). In the non-irradiated fresh serum medium both the low concentration (1×10^{-10} M) of all-trans-RA and the high concentration (1×10^{-7} M) of the all-trans-RA revealed negligible to moderate stimulatory effect on the incorporation (biosynthesis) of free [^{14}C]inositol into PtdIns4,5P_2 when compared to control incubations that contained no retinoids (Fig 3*A*). However, when similar experiments were conducted in a medium supplemented with UV-irradiated serum, incubation with low all-trans RA concentration (1×10^{-10} M) had little effect. On the other hand, the high RA concentration (1×10^{-7} M), which revealed no striking stimulatory effort at 12 and 24 h, respectively,

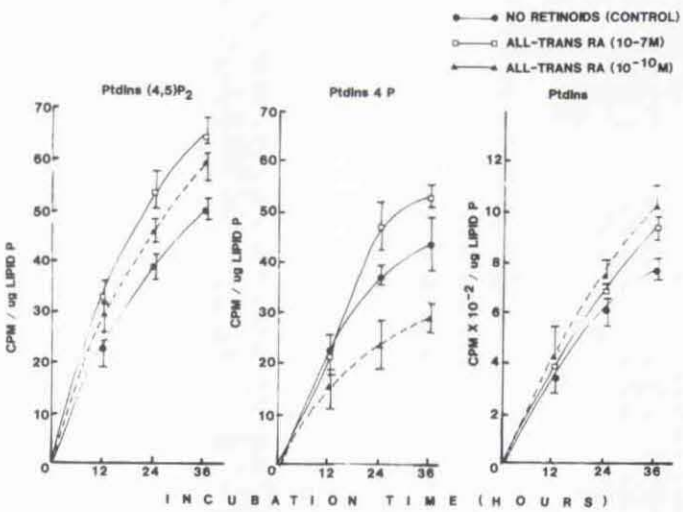

Figure 2. Dose-dependent Effects of All-trans Retinoic Acid on the Incorporation of [^{14}C] Inositol into Keratinocyte Inositol Phospholipids Cultured in a Fresh Serum (Retinol-Containing) Medium. Keratinocytes (6×10^6/60 mm plate) in 3 ml of DMEM, which is supplemented with fresh 10% FCS (retinol-containing non-irradiated), were incubated at 37°C with 0.4 μCi of myo-(U-^{14}C)-inositol (53.3 mCi/mol) in the presence of either a high (1×10^{-7} M) or a low (1×10^{-10} M) concentration of all-trans RA. Each incubation contained LiCl (10 mM). Controls were duplicate keratinocyte incubations in the absence of all-trans-RA. Radioactivity incorporated into the ^{14}C-inositol phospholipids was determined after chromatography on the TLC plates as described in the *Methods* Section. Results are expressed as counts per minute/microgram of phosphorus in the extracted total inositol phospholipids (cpm/μg lipid P). Each point represents the mean ±S.D. of duplicate incubations from two separate experiments. The stimulation of ^{14}C-inositol incorporation into PtdIns4,5P_2 ($p < 0.005$), PtdIns4P ($p < 0.003$), and PtdIns ($p < 0.005$) by high concentration of all-trans-RA (1×10^{-7} M) at 36 h is statistically significant when compared to non-retinoid-treated cells.

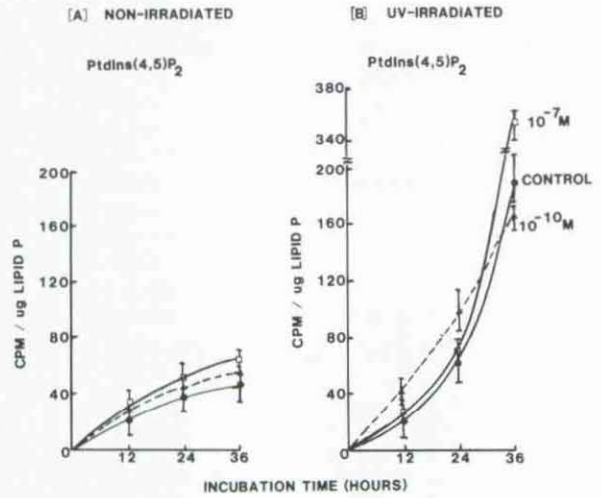

Figure 3. Comparative Effects of High and Low Concentrations of All-trans-RA on the Incorporation of ^{14}C-Inositol into Keratinocyte [^{14}C]PtdIns4,5P_2 Cultured in either Retinol-free or Retinol-containing Medium. Keratinocytes (6×10^6/60 mm plate) were incubated with 0.4 μCi of myo-(U-^{14}C)-inositol in a medium supplemented with either a retinol-free UV-irradiated calf serum (*B*) or a medium supplemented with retinol-containing fresh calf serum (*A*) as described in the legend of Fig 2. The incubations were treated with either high (1×10^{-7} M) or low (1×10^{-10} M) all-trans-RA. Controls were duplicate keratinocyte incubations in the absence of all-trans-RA. Radioactivity incorporated into the ^{14}C-PtdIns4,5P_2 was determined after chromatography on the TLC plates as described in the *Methods* Section. Results are expressed as counts per min/μg of phosphorus in the extracted total inositol phospholipids (cpm/μg lipid P). Each point represents the mean ±S.D. of duplicate incubations from two separate experiments. Incorporation of ^{14}C-inositol into PtdIns4,5P_2 of keratinocytes cultured in medium containing UV-irradiated serum (*B*) is enhanced by high concentration of all-trans-RA (1×10^{-7} M) at 36 h. This increase is statistically significant ($p < 0.0015$) when compred to cells cultured in a medium containing non-irradiated serum (*A*).

revealed a significant ($P < 0.0015$) incorporation of ^{14}C-inositol into PtdIns4,5P_2 at 36 h (Fig 3*B*). In additional control experiments, non-radioactive retinyl acetate (1×10^{-8} M), was added back to a medium previously supplemented with UV-irradiated serum (retinol free) which contained keratinocytes. When these cells in culture were challenged by either of the retinoids at a low concentration (1×10^{-10} M), transient release of ^{14}C-InsP_3 was negligible, a response similar to results from the unirradiated serum (data not shown). Data from these experiments underscore that in order to obtain full information on the concentration-dependent effects of natural all-trans retinoic acid on keratinocytes grown in a medium containing fresh serum, it will be desirable to carry out similar incubations in a non-serum (retinol) containing medium.

Short-term Effect of a Low Concentration of All-trans-RA on the Hydrolysis of Keratinocyte [^{14}C]PtdIns4,5P_2 The negligible effect of low concentration of all-trans-RA on the incorporation of ^{14}C-inositol into ^{14}C-PtdIns4,5P_2, prompted us to explore whether a similar or different effect of this low physiologic concentration is exerted on the hydrolysis of the labeled keratinocyte PtdIns4,5P_2. In an initial experiment, the incubation of previously labeled keratinocytes with a low concentration of the natural all-trans retinoic acid (1×10^{-10} M) resulted in a rapid and enhanced hydrolysis of keratinocyte ^{14}C-PtdIns4,5P_2 when compared to non-retinoid containing control (Fig 4*A*). In subsequent experiments, hydrolysis of ^{14}C-PtdIns4,5P_2 was accompanied by a statistically significant ($p < 0.005$) rapid and transient release of [^{14}C]InsP_3 (Fig 4*B*). These data demonstrate that low concentration of all-trans-RA, which exerted an insignificant effect on the incorporation of ^{14}C-inositol into PtdIns4,5P_2 (biosynthesis), does produce a significant and rapid effect on the hydrolysis of ^{14}C-PtdIns4,5P_2 from prelabeled cultured keratinocytes when compared to non-retinoic acid controls. The reason for the slow increase in the release of the inositol triphosphate (beyond 60 sec), which is evident in the non-RA treated control, is presently unclear. In a separate experiment, incubation with a high concentration (1×10^{-5}M) of all-trans-RA suppressed hydrolysis of PtdIns4,5P_2.

Comparative Effects of All-trans-RA and 13-Cis RA on the Hydrolysis of Keratinocyte [^{14}C]PtdIns4,5P_2 We investigated whether a difference exists between the in vitro effects of a low concentration of all-trans-RA and its 13-Cis-RA analog on the hydrolysis of keratinocyte ^{14}C-PtdIns4,5P_2. To accomplish this, low concentrations (1×10^{-10} M) of all-trans-RA and its 13-Cis analog were incubated with prelabeled keratinocytes in the preferable medium supplemented with UV-irradiated (retinol-free) serum as described above. Data from a time-dependent hydrolysis of [^{14}C]PtdIns4,5P_2 revealed a rapid and enhanced hydrolysis of [^{14}C]PtdIns4,5P_2 by both all-trans-RA and 13-Cis-RA with maximal activity at 30 secs (Fig 5*A*) when compared to non-retinoid containing control. Although the hydrolysis of PtdIns4,5P_2 was enhanced by the natural all-trans-RA as shown previously, the stimulatory effect of its analog (13-Cis retinoic acid) was more marked.

Furthermore, hydrolysis of [^{14}C]PtdIns4,5P_2 by both retinoids was accompanied by a rapid "transient" generation of water soluble [^{14}C]InsP_3 (Fig 5*B*) with maximal accumulation at 30 sec. Similarly, the effect of 13-Cis-RA on the generation of ^{14}C-InsP_3 was strikingly higher than the all-trans-RA.

In parallel experiments we compared the effects of a high concentration (1×10^{-5} M) and a low (1×10^{-10} M) concentration of

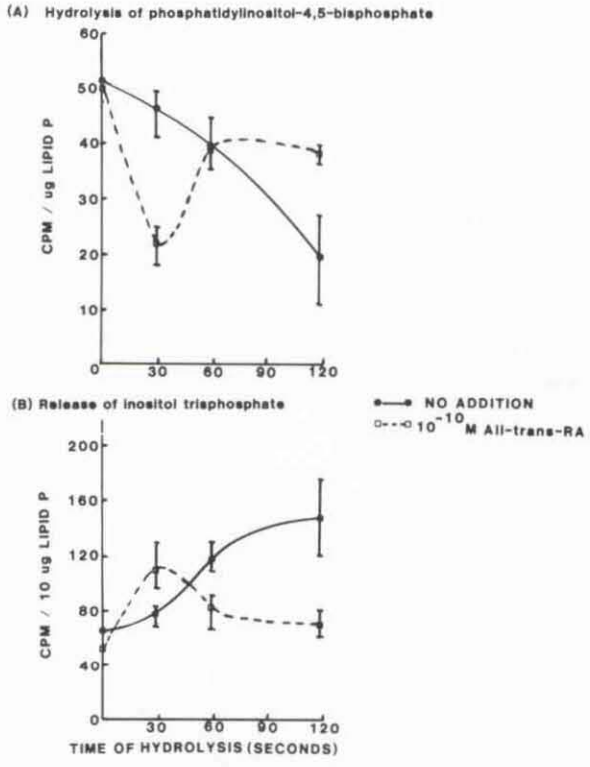

Figure 4. Short-term Effects of a Low Concentration of All-trans-RA on the Hydrolysis of Keratinocyte [^{14}C]PtdIns4,5P_2. Keratinocytes (6×10^6/60 mm plate) were pre-labeled and cultured as described in Fig 2. Culture plates were replenished with serum previously irradiated at 254 nm as described in the text. Hydrolysis of cellular [^{14}C]PtdIns4,5P_2 and generation of ^{14}C-InsP_3 were initiated with or without the addition of all-trans RA. Time-course and identification of products are as indicated in Fig. 2. Results are expressed as cpm/μg total lipid phosphorus. Each point represents the mean ±S.D. of duplicate incubations from two separate experiments. Low concentration (1×10^{-10} M) of all-trans-RA hydrolysis of PtdIns4,5P_2 [A] and release of InsP_3 [B] at 30 secs were statistically significant, ($p < 0.005$) and ($p < 0.005$), respectively, when compared to non-retinoid-treated cells.

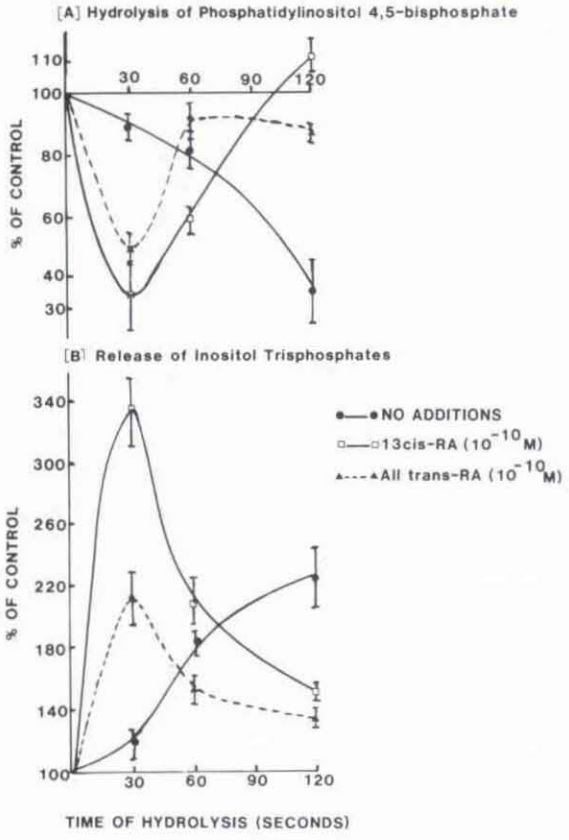

Figure 5. Comparative Effects of All-trans-RA and 13-Cis-RA on Hydrolysis of Keratinocyte [^{14}C]PtdIns4,5P_2. Keratinocytes (6×10^6/60 mm plate) were prelabeled and cultured as described in Fig 2. Culture plates were replenished with serum previously irradiated at 254 nm as described in the text. Hydrolysis of cellular [^{14}C]PtdIns4,5P_2 and generation of ^{14}C-InsP_3 were initiated with or without the addition of all-trans-RA or 13-Cis RA to the culture plate. Time course and identification of products are as indicated in Fig 2. Results are expressed as percentage of non-hydrolyzed ^{14}C-PtdIns4,5P_2 after normalization at 100%. Similarly, InsP_3 generated is expressed as percentage over control (normalized at 100%). Each point represents the mean ±S.D. of duplicate incubations from two separate experiments. Hydrolysis of ^{14}C-PtdIns4,5P_2 (Top, *A*) at 30 secs by 13-Cis RA ($p < 0.005$) and all-trans RA ($p < 0.005$) are statistically significant when compared to non-retinoid-treated cells. Similarly, the generation of ^{14}C-InsP_3 (*Lower* Fig 5*B*) at 30 secs by 13-Cis-RA ($p < 0.0005$) and all-trans-RA ($p < 0.005$) are statistically significant when compared to non-retinoid-treated cells.

13-Cis-RA on the generation of ^{14}C-InsP_3 from ^{14}C-PtdIns4,5P_2 of prelabeled cells. Two controls for these studies included incubations with low exogenous concentration of retinal (1×10^{-10} M) and incubations with no added retinoids. We observed a rapid and enhanced transient generation of ^{14}C-InsP_3 by the low concentration (1×10^{-10} M) of 13-Cis-RA when compared to the two control incubations, which contained either retinal or no retinoids (Fig 6). Interestingly, a high concentration (1×10^{-5} M) of 13-Cis-RA did not enhance the generation of ^{14}C-InsP_3 but rather induced a moderate suppression of ^{14}C-InsP_3 generation.

DISCUSSION

Results from these studies clearly demonstrate that all-trans-RA and its 13-Cis analog exert dose-dependent effects on the turnover of inositol phospholipids of cultured murine keratinocytes. A moderately high concentration (1×10^{-7} M) of either retinoid exerts varying increases on the biosynthesis of the inositol phospholipids during the first 36 h of keratinocytes in culture, while the effects with a low physiologic concentration (1×10^{-10} M) are minor and insignificant. The observed stimulatory effect of the retinoids on the biosynthesis of the inositol phospholipids is interesting because these cells in the early growth phase in culture are rapidly proliferating. High concentrations of the retinoids possibly alter this proliferative activity and thus function to stimulate the increased biosynthesis of the inositol phospholipids. These increased inositol phospholipids are required lipid constituents that serve as membrane building blocks for the rapidly proliferating new cells. Interestingly, 13-Cis-RA, an analog of the all-trans-RA, is more effective than the natural all-trans-RA at the same high concentration in the in vitro stimulation of inositol phospholipid biosynthesis of these proliferating cells.

In contrast to the insignificant stimulatory effects of low physiologic concentration (1×10^{-10} M) of both all-trans-RA and its 13-Cis analog on the incorporation of free inositol into the more phosphorylated PtdIns4,5P_2, both retinoids at low concentrations rapidly and significantly enhanced the hydrolysis of the keratinocyte ^{14}C-PtdIns4,5P_2 in vitro (Fig 5*A*). This hydrolysis was accompanied by a simultaneous "transient" generation of ^{14}C-InsP_3 (Fig 5*B*). These very early and rapid biochemical events by the two forms of retinoids are novel and do precede the onset of detectable evidence of differentiation in these cells. These results imply that the in vitro effect of low physiologic concentrations, and not the high concentration of these two forms of retinoids on cultured keratinocytes, may be directed at least in part towards the initiation of an early hydrolysis of PtdIns4,5P_2 resulting in a rapid, transient generation of InsP_3. Although not measured in these studies, it is likely that diacylglycerol (DAG) was also released as shown in the speculative scenario of retinoic acid-induced generation of InsP_3 from PtdIns4,5P_2 (Fig 7). The early release of these intracellular "second messengers" may function as the initial signal which in turn may act alone or in concert with other modulators to program the proliferating cells into differentiation.

Although our present data are inadequate to directly implicate InsP_3 with exerting the intracellular stimulus activity that initiates the course of keratinocyte differentiation, the enhancement of the hydrolysis of the keratinocyte PtdIns4,5P_2 coupled with the rapid, transient generation of InsP_3, is consistent with agonist-induced rapid breakdown of PtdIns4,5P_2 and accumulation of InsP_3 as re-

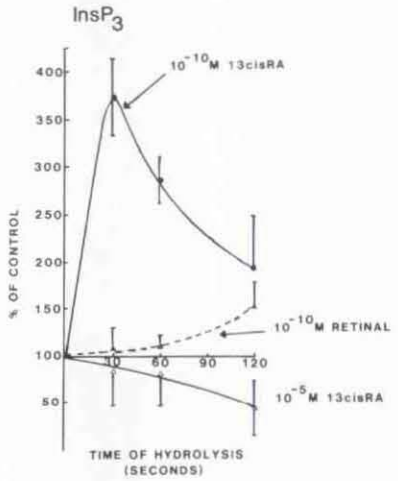

Figure 6. Short-term Effects of High and Low Concentrations of 13-Cis-RA on the Generation of ^{14}C-InsP_3 from Keratinocyte [^{14}C]PtdIns4,5P_2. Keratinocytes (6×10^6/60 mm plate) were prelabeled and cultured as described in Fig 2. Culture plates were replenished with serum previously irradiated at 254 nm as described in the text. Hydrolysis of cellular [^{14}C] PtdIns4,5P_2 and generation of ^{14}C-InsP_3 were initiated with or without the additions of 13-Cis RA and retinal. Time-course and identification of products are as indicated in Fig 2. ^{14}C-InsP_3 generated is expressed as percentge over control (normalized at 100%). Each point represents the mean ±S.D. of duplicate incubations from two separate experiments. Release of InsP_3 by 13-Cis-RA ($p < 0.0005$) at low concentration (1×10^{-10} M) is statistically significant when compared to a high concentration of 13-Cis-RA (1×10^{-5} M), non-active retinal (1×10^{-10} M), or non-retinoid treated cells.

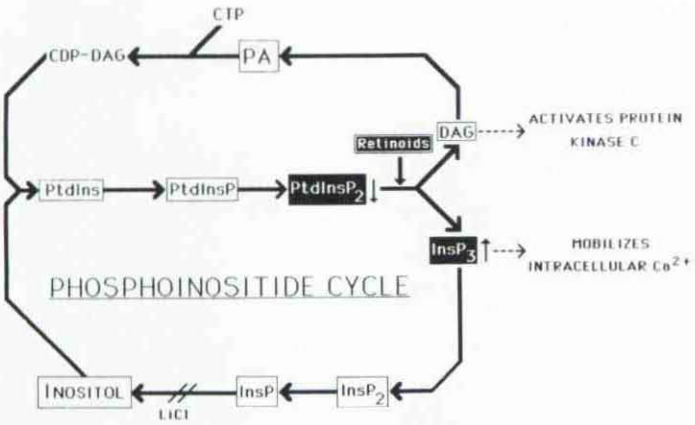

Figure 7. Speculative Scenario of the Retinoid-induced Hydrolysis of PtdIns4,5P_2 and Generation of Inositol Triphosphate.

ported in other systems [17,18,25]. Furthermore, the generation of $InsP_3$ and the release of intracellular Ca^{2+} have been reported in a number of cell types. Strong evidence that $insP_3$ may be a putative messenger comes from studies showing that $InsP_3$ releases Ca^{2+} from permeabilized cells [26–28]. The direct regulatory role of $InsP_3$ in keratinocyte intracellular Ca^{2+} release and differentiation remains to be determined. Nonetheless, the all-trans-RA and its 13-Cis-analog induced-release of $InsP_3$ in the present studies is consistent with biochemical events previously observed in a recent study from our laboratory [22]. This study demonstrated a rapid, transient generation of $InsP_3$ prior to the onset of keratinocyte differentiation. Although the present data do not delineate the precise mode of action of the two forms of retinoic acid used in these studies, one possibility is the presence in keratinocytes of retinoic acid-binding protein, which has been reported in a number of tissues [19], including chick embryo skin [20]. Alternatively, vitamin A (retinol) has been shown to interact with biologic membranes [29,30]. These interactions have led to the suggestion that a physiologic concentration of the retinol may perturb the cellular lipoprotein membranes, thus initiating hydrolysis of the cellular PtdIns4,5P_2 and generation of $InsP_3$. Further experiments are, however, necessary to substantiate this membrane-active receptor-mediated possibility in the keratinocytes. If so, then the phenomena observed in these studies will be consistent with other agonist receptor-induced hydrolysis of the cellular PtdIns4,5P_2, the generation of $InsP_3$, and thus modulation of cellular events that regulate keratinocyte differentiation.

The excellent secretarial assistance of Ms. Georgia Brown and the expert photo-illustrations by Ms. Irene Rowley are gratefully acknowledged.

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